Chitin is present within the Ampullae of Lorenzini of chondrichthyan fishes

Molly Phillips[1,2], W. Joyce Tang[3], Khan Hassan[2], Daniel Ocampo Daza[2,4], Murat Kaya[5], Idris Sargn[6], and Chris T. Amemiya[2,1,*]

[1] Department of Biology, University of Washington, Seattle, WA 98195, USA.

[2] Department of Molecular and Cell Biology, University of California, Merced, Merced, CA 95343, USA.

[3] Department of Orthopaedics and Sports Medicine, University of Washington School of Medicine, Seattle, WA 98109, USA.

[4] Department of Organismal Biology, Uppsala University, 75236 Uppsala, Sweden

[5] Department of Biotechnology and Molecular Biology, Aksaray University, 68100 Aksaray, Turkey

[6] Department of Biochemistry, Faculty of Science, Selcuk University, 42075 Konya, Turkey

[*] To whom correspondence should be addressed (camemiya@ucmerced.edu)

ABSTRACT

Chitin is produced by a variety of organisms using enzymes called chitin synthases and was recently discovered in certain vertebrates. In our ongoing investigations into the presence of vertebrate chitin, we unexpectedly found it within the electrosensory organs, known as Ampullae of Lorenzini, of diverse cartilaginous fishes. We used histochemical reagents, chemical analyses, and enzymatic digestions to confirm our findings. Further, *in situ* hybridization with a sequence from the little skate (*Leucoraja erinacea*) revealed that chitin synthase expression is localized to cells inside the organs. These findings indicate that cartilaginous fishes endogenously synthesize chitin and beg further investigation into the function of chitin in the electrosensory system.

INTRODUCTION

We previously reported that the polysaccharide, chitin, a key component of arthropod exoskeletons and fungal cell walls, is endogenously produced by fishes and amphibians in spite of the widely held view that it was not synthesized by vertebrates [1]. Genes encoding chitin synthase enzymes were found in the genomes of a number of fishes and amphibians and shown to be correspondingly expressed at the sites where chitin was localized [1, 2]. In this report, we show that chitin is prevalent within the specialized electrosensory organs of cartilaginous fishes (Chondrichthyes). These organs, the Ampullae of Lorenzini (AoL), are found ventrally and dorsally and are most concentrated toward the rostral end of the animals [3]. The AoL comprise a series of gel-filled canals emanating from surface pores in the skin (Fig. 1A) which extend into bulbous structures (alveoli) containing sensory cells capable of detecting subtle changes in electric fields (Fig. 1B) [4].

Our findings extend the number of vertebrate taxa where endogenous chitin production has been detected and raise many questions regarding its structural composition and function within the class Chondrichthyes and other aquatic vertebrates.

RESULTS

Affinity histochemistry was carried out with chitin probes [1] on formalin-fixed little skate (*Leucoraja erinacea*) embryos (Fig. 1C,D). The results revealed an overt correspondence of fluorescent chitin signals with AoL structures. Further experiments using confocal microscopy demonstrated fluorescent signal throughout the AoL from the pore to the alveolus (Fig. 1E). Chitin is localized to the acellular lumen of AoL canals and is a component of the viscous hydrogel (Figs. 1F, S1A). The space-filling nature of the chitinous hydrogel within the canal lumen of an adult shark and skate can be visualized in Fig. S1A and B, respectively.

We investigated and validated the existence of AoL chitin using multiple methods. First, we used chitin-digesting enzymes (chitinases) on AoL-containing histological sections. When chitinase was applied to the sections, we observed the elimination of detectable chitin (Fig. 1G) compared to controls (Fig. 1F). This was done with AoL-containing tissues from multiple chondrichthyan species. Likewise, chitinase digestion of a colloidal solution of raw AoL hydrogel from a big skate (*Raja binoculata*) resulted in a marked increase of chitinolytic products relative to no-enzyme controls (Supplement). We also chemically extracted polysaccharides from big skate hydrogel for analysis by FTIR (Fourier transform infrared spectroscopy). The resulting spectrum showed characteristic chitin peaks (Fig. S2). Chitin exists in three main allomorphic forms (α-, β-, γ-) [5] and by

comparing the AoL spectrum to those of known α- and β-chitin controls, we observe a pattern most closely resembling that of β-chitin. This is consistent with the fact that β-chitin is usually associated with softer, more pliable structures than α-chitin [5]. Lastly, we used two unrelated histochemical probes to detect chitin in whole-mount and histological preparations of multiple chondrichthyan species, including the spotted ratfish, *Hydrolagus colliei* (Fig. S1D, E). Both probes yielded highly similar patterns despite their disparate amino acid sequences [1].

In order to verify that the chitin within the AoL was indeed generated by endogenous chitin synthase activity, we used antisense riboprobes for *in situ* hybridization (ISH) on both whole-mount (Fig. 1H, I) and sectioned material (Fig. 1J,K) of embryonic *L. erinacea*. The whole-mount ISH patterns using the *LeCHS* riboprobe (Supplement) indicated expression at the AoL pores (Fig. 1H), and chitin seems to be intimately associated with these sites (Fig. 1I). The ISH signals in this particular experiment were restricted to the pore sites, however, we have observed that probe penetration can be variable likely due to the fish's thick skin. We thus used sectioned materials and found *LeCHS* signals in the epithelial cells lining AoL canal walls at various canal depths (Figs. 1J, S1C). Additionally, the ISH signals in Fig. S1C, which are cross sections of AoL at different levels and transverse angles, indicate that cells inside the alveoli do not exhibit appreciable chitin synthase gene activity.

DISCUSSION

In this paper, we show that chitin is found within the AoL of cartilaginous fishes. The taxa which have thus far been found to exhibit chitin in their AoL are given in Fig. S1F and

are representative of both elasmobranch and holocephalan lineages. The presence of chitin within the AoL of both subclasses (and in diverse orders within the Elasmobranchii) suggests that this condition evolved in the ancestor to all cartilaginous fishes, perhaps in the placoderms [6]. A more detailed treatment of chitin synthase genes within Chondrichthyes will be reported elsewhere.

The Ampullae of Lorenzini are sensory organs that detect weak electric fields at the skin surface and transduce them into neuronal signals that project onto the brain. The subsequent topographical map of the organism's electrical surroundings are thought to be used for various biological functions, most notably the detection of prey, localization of conspecifics for mating, and navigation [4]. While the electrophysiology of voltage-gated ion channels in the alveolar electrosensory cells has been the subject of active investigation, the actual physico-chemical mechanism by which the signals are first detected at the pores and traverse through the hydrogel within the canals still remains to be worked out [7, 8]. We and our collaborators have previously shown that the AoL hydrogel is extremely proton-conductive [9], and that this property may putatively contribute to the electrosensing mechanism by providing an electrical environment that is markedly more conductive than the surrounding seawater. The role of chitin in this overall scheme is enigmatic as it does not have a chemical structure that is conductive and it is insoluble in aqueous solutions. However, by forming a composite material it may contribute to the gel's space-filling properties (Fig. S1A,B) and serve as an insulating scaffold for the attachment of proteins and sulfated glycosaminoglycans (proteoglycans) that are also components of the gel [10]. Regardless, our new findings expand the scope of vertebrate chitin to the Chondrichthyes and raise many questions with respect to the

overarching uses and properties of this versatile biopolymer and why it has been evolutionarily lost in the land-dwelling vertebrates.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, supplemental experimental procedures and supplemental references.

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AUTHOR CONTRIBUTIONS

M.P., W.J.T., K.H. and C.T.A. conceived the experiments; M.P., W.J.T., K.H., D.O.D., M.K., I.S., and C.T.A. collected the data and performed the analyses. M.P. and C.T.A. wrote the paper with input from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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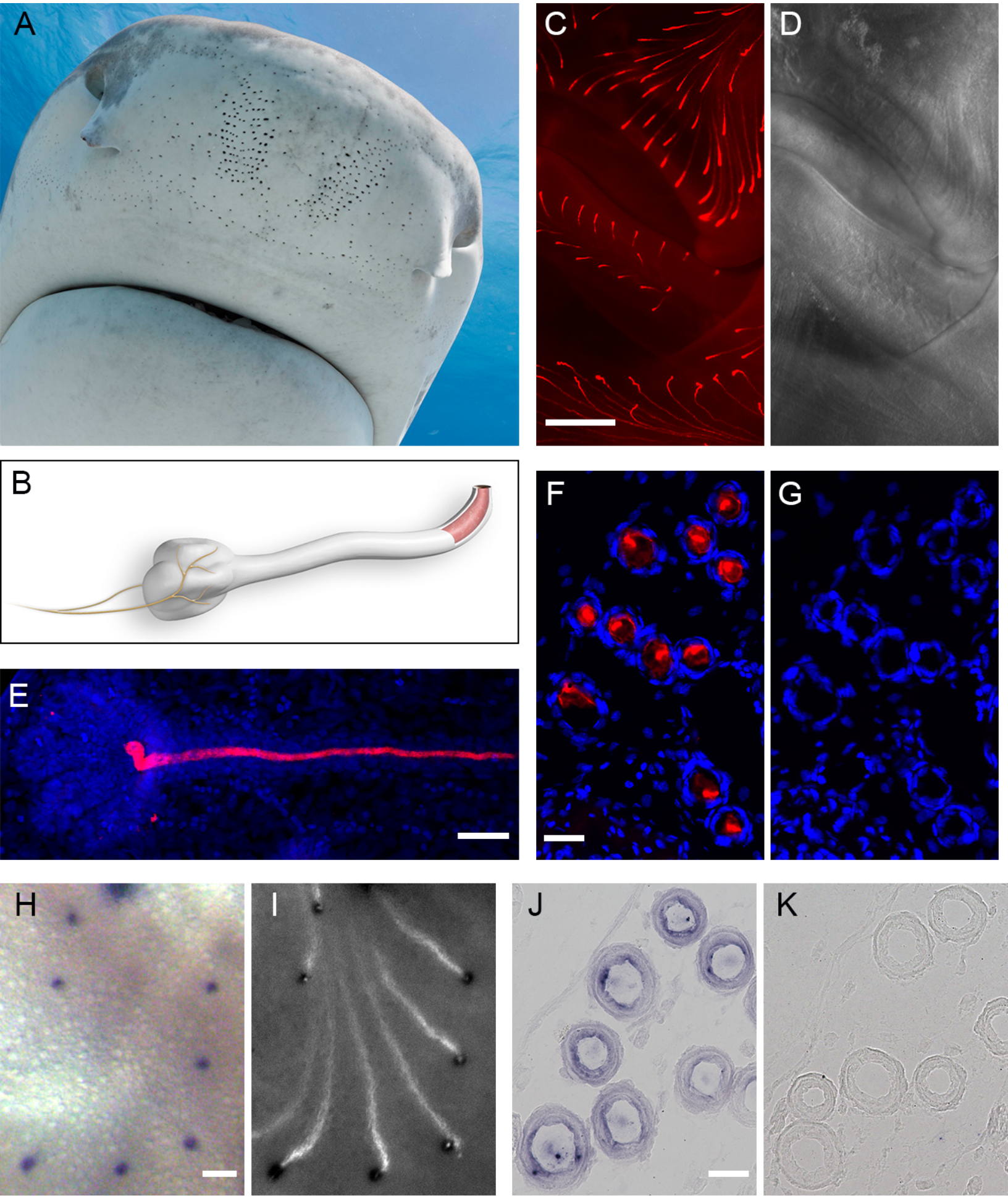
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Figure 1. Description of Ampullae of Lorenzini and characterization of the AoL chitin. (A) Snout of a tiger shark (*Galeocerdo cuvier*) revealing its numerous AoL pores. Photo taken by Neil Hammerschlag. (B) Illustration depicting an individual AoL. The pore at the upper right leads into a canal encased by a collagenous sheath filled with an acellular hydrogel (red) as depicted by the cutaway region. At the distal end of the organ is a round, lobed structure, the alveolus. The alveolus contains hundreds or thousands of electrosensory cells that synapse with afferent neurons and transmit information to the brain and which, collectively, make a topographical image of the surrounding electrical environment. (C, D) Whole-mount preparation of an embryonic little skate (*Leucoraja erinacea*) labeled with a fluorescent (red) chitin probe (C) and the corresponding brightfield image of the same specimen (D). The fluorescence pattern directly correlates with the distribution of AoL (raised canals on the surface of the skin). This field is the ventral surface around the lower lip (the middle band of AoL in C). (E) Confocal image of a chitin-labeled AoL from an embryonic catshark (*Scyliorhynus canicula*). Nuclei are stained with DAPI (blue) and delineates the canal and alveolus, and chitin (red) is seen throughout and across the canal. (F, G) *in situ* chitinase assay of AoL of embryonic *L. erinacea*. (F) Adjacent paraffin cross sections of wing tissue were stained for chitin (red) after treatment with buffer (F) or chitinase (G), then counterstained with DAPI (blue). Chitin is seen within the AoL lumen of buffer-only controls (F) but not the chitinase-digested sample (G). (H-K) Confirmation of chitin synthase gene expression in the AoL by *in situ* hybridization (ISH). A skate antisense riboprobe (*LeCHS*) was used for ISH on whole-mount (H,I) or paraffin sections (J) of embryonic *L. erinacea*. The riboprobe (blue) localized to regions in the vicinity of the pores of the AoL (H), which were corroborated by chitin histochemistry

(white) of the same tissue specimen (I). (J) Chitin synthase gene expression is seen in the endothelial cells lining the canal. (K) ISH negative control. Scale bars are 500 μm (C, D), 50 μm (E), 20 μm (F, G), 50 μm (H, I), and 10 μm (J, K).